

Amendments to the Specification

Please replace the current sequence listing with the Substitute Sequence Listing, appended hereto, at the end of the application.

Please replace the paragraph bridging pages 16-17 with the following:

The choice of cleavage site may have a bearing on the precise nature of the N-terminus (or C-terminus) of the released polypeptide. To illustrate this, identical LH_N/B fragments produced in such proprietary systems are described in SEQ ID 88, 94, 96, 98, in which the N-terminal extensions to the LH_N/B sequence are ISEFGS (SEQ ID NO: 192), GS, SPGARGS (SEQ ID NO: 193) & AMADIGS (SEQ ID NO: 194) respectively. In the case of LH_N/C fragments, SEQ ID 126, 128 & 130 describe the N-terminal sequences VPEFGSSRVDH (SEQ ID NO: 195), ISEFGSSRVDH (SEQ ID NO: 196) and VPEFGSSRVDH (SEQ ID NO: 197) following release of the LH_N/C fragment from its fusion tag by enterokinase, genenase and Factor Xa respectively. Each of these extension peptide sequences is an example of a variant L-chain sequence of the present invention. Similarly, if the purification tag were to be fused to the C-terminal end of the second domain, the resulting cleaved polypeptide (ie. fusion protein minus purification tag) would include C-terminal extension amino acids. Each of these extension peptides provides an example of a variant H_N portion of the present invention.

Please replace the paragraph bridging pages 17-18 with the following:

Depending on the cleavage enzyme chosen, this strategy may result in a short amino acid extension to the N-terminus (or C-terminus) of the polypeptide. For example,

in the case of SEQ ID 92, cleavage of the expressed product with enterokinase results in two polypeptides coupled by a single disulphide bond at the first domain-second domain junction (ie. the L chain-H_N junction), with a short N-terminal peptide extension that resembles an intact Factor Xa site and a short N-terminal extension due to polylinker sequence (IEGRISEFGS) (SEQ ID NO: 198).

Please replace the first full paragraph on page 18 with the following:

Secondly, the DNA encoding a self-splicing intein sequence may be employed, which intein may be induced to self-splice under pH and/or temperature control. The intein sequence (represented in SEQ ID 110 as the polypeptide sequence ISEFRESGAISGDSLISLASTGKRVSIDLLDEKDFEIWAINETQTMKLESAKVSRVFCTGKKLVYILKTRLGRTIKATANHRFLTIDGWKRLDELSLKEHIALPRKLESSSLQLSPEIEKLSQSDIYWDSIVSITETGVVEVFDLTVPGPHNFVANDIIVHN (SEQ ID NO: 199)) facilitates self-cleavage of the illustrated polypeptide (ie. purification tag-LH_N/B) to yield a single polypeptide molecule with no purification tag. This process does not therefore require treatment of the initial expression product with proteases, and the resultant polypeptide (ie. L-chain-Factor Xa activation site - H_N) is simply illustrative of how this approach may be applied.

Please replace Table 2 on page 20 with the following:

Table 2 - spacer sequences

<u>Sequence</u>	<u>Illustrated in SEQ ID No</u>
(GGGGS) ₃	39/40, 43/44, 49/50, 53/54, 57/58
RNaseA loop	138/139
Helical	41/42, 45/46, 47/48, 51/52, 55/56

Att sites (TSLYKKAGFGS (SEQ ID NO: 200) 133
or DPAFLYKV (SEQ ID NO: 201))

Please replace the paragraph bridging pages 21-22 with the following:

First, DNA encoding the molecular clamp has been ligated directly to the DNA encoding an LH_N polypeptide, after removing the STOP codon present in the LH_N coding sequence. By insertion, to the 3' of the LH_N sequence, of overlapping oligonucleotides encoding the clamp sequence and a 3' STOP codon, an expression cassette has been generated. An example of such a sequence is presented in SEQ ID 63 in which the DNA sequence coding for the molecular clamp known as fos (LTDTLQAETDQLEDEKSALQTEIANLLKEKEKLEFILAAH) (SEQ ID NO: 202) has been introduced to the 3' of a nucleic acid molecule encoding a LH_N/A polypeptide, which molecule also has a nucleic acid sequence encoding an enterokinase cleavage site between the coding regions of the first domain (L-chain) and the second domain (H_N).

Please replace the first full paragraph on page 22 with the following:

Secondly, site-specific recombination has been utilised to incorporate a clamp sequence to the 3' of a LH_N polypeptide (see, for example, the ~~GATEWAY~~ GATEWAY[®] cloning system described below) spaced from the H_N domain by the short peptide Gly-Gly. Use of this peptide to space clamp sequences from the C-terminus of H_N is illustrated in SEQ 117/118.

Please replace the third paragraph on page 23 with the following:

To help illustrate this point, several TeNT based polypeptides have been prepared according to the present invention, and reference is made to SEQ ID 143 which is an LH_N polypeptide having a C-terminal sequence of EEDIDV₈₇₉ (SEQ ID NO: 207). Reference is also made to SEQ ID 147 which is an LH_N polypeptide having a C-terminal sequence of EEDIDVILKKSTIL₈₈₇ (SEQ ID NO: 203). Both of these LH_N sequences are representative of 'native' TeNT LH_N sequences, which have no introduced specific cleavage site between the L-chain and the H_N domain. Thus, SEQ ID 145 illustrates a TeNT polypeptide according to the present invention in which the natural TeNT linker region between the L-chain and the H_N domain has been replaced with a polypeptide containing a specific enterokinase cleavage sequence.

Please replace the paragraph bridging pages 28-29 with the following:

Example 4 *et seq* illustrate a number of alternative conventional methods for engineering recombinant DNA molecules that do not require traditional methods of restriction endonuclease-dependent cleavage and ligation of DNA. One such method is the site-specific recombination ~~GATEWAY (trade mark)~~ GATEWAY[®] cloning system of Invitrogen, Inc., which uses phage lambda-based site-specific recombination [Landy, A. (1989) Ann. Rev. Biochem. 58, pp. 913-949]. This method is now described in slightly more detail.

Please replace the first two full paragraphs on page 29 with the following:

Using standard restriction endonuclease digestion, or polymerase chain reaction techniques, a DNA sequence encoding first and second domains (e.g. a BoNT LH_N

molecule) may be cloned into an ~~Entry-Vector~~ ENTRY VECTOR (cloning vector). There are a number of options for creation of the correct coding region flanked by requisite *att* site recombination sequences, as described in the ~~GATEWAY (trade mark)~~ GATEWAY[®] (cloning system) manual.

For example, one route is to insert a generic polylinker into the ~~Entry-Vector~~ ENTRY VECTOR (cloning vector), in which the inserted DNA contains two *att* sites separated by the polylinker sequence. This approach facilitates insertion of a variety of fragments into the ~~Entry-Vector~~ ENTRY VECTOR (cloning vector), at user-defined restriction endonuclease sites.

Please replace the last two contiguous paragraphs on page 29 with the following:

Examples of ~~Entry-Vectors~~ ENTRY VECTORS (cloning vectors) are provided for LH_N/C (SEQ ID 135), for LH_N/C with no STOP codon thereby facilitating direct fusion to ligands (SEQ ID 136), and for a L-chain/C sequence that can facilitate combination with an appropriate second or third domain (SEQ ID 134).

By combination of the modified ~~Entry-Vector~~ ENTRY VECTOR (cloning vector) (containing the DNA of interest) and a ~~Destination-Vector~~ DESTINATION VECTOR (cloning vector) of choice, an expression clone is generated. The ~~Destination-Vector~~ DESTINATION VECTOR (cloning vector) typically provides the necessary information to facilitate transcription of the inserted DNA of interest and, when introduced into an appropriate host cell, facilitates expression of protein.

Please replace the first two full contiguous paragraphs on page 30 with the following:

~~Destination Vectors~~ DESTINATION VECTORS (cloning vectors) may be prepared to ensure expression of N-terminal and/or C-terminal fusion tags and/or additional protein domains. An example of a novel engineered ~~Destination Vector~~ DESTINATION VECTOR (cloning vector) for the expression of MBP-tagged proteins in a non-transmissible vector backbone is presented in SEQ ID 137. In this specific embodiment, recombination of an ~~Entry Vector~~ ENTRY VECTOR (cloning vector) possessing a sequence of interest with the ~~Destination Vector~~ DESTINATION VECTOR (cloning vector) identified in SEQ ID 137 results in an expression vector for *E. coli* expression.

The combination of ~~Entry~~ ENTRY VECTORS (cloning vectors) and ~~Destination Vectors~~ DESTINATION VECTORS (cloning vectors) to prepare an expression clone results in an expressed protein that has a modified sequence. In the Examples illustrated with SEQ ID 30 & 124, a peptide sequence of TSLYKKAGF (SEQ ID NO: 204) is to be found at the N-terminus of the endopeptidase following cleavage to remove the purification tag. This peptide sequence is encoded by the DNA that forms the *att* site and is a feature of all clones that are constructed and expressed in this way.

Please replace the fourth paragraph on page 30 with the following:

It will be also appreciated that the precise N-terminus of any polypeptide (eg. a LH_N fragment) will vary depending on how the endopeptidase DNA was introduced into the ~~entry vector~~ ENTRY VECTOR (cloning vector) and its relationship to the 5' *att* site.

SEQ ID 29/30 & 123/124 are a case in point. The N-terminal extension of SEQ ID 30 is TSLYKKAGFGS (SEQ ID NO: 205) whereas the N-terminal extension of SEQ ID 124 is ITSLYKKAGFGSLDH (SEQ ID NO: 206). These amino acid extension-containing domains provide further examples of first/second domain variants according to the present invention.

Please replace the first full paragraph on page 39 with the following:

29 DNA coding for LH_N/A as expressed from a Gateway GATEWAY[®] (cloning system) adapted ~~destination vector~~ DESTINATION VECTOR (cloning vector). LH_N/A incorporates an enterokinase activation site at the LC-H_N junction and an 11 amino acid *att* site peptide extension at the 5' end of the LH_N/A sequence

Please replace the four contiguous paragraphs, beginning with the second full paragraph on page 57 and extending to page 58 with the following:

134 DNA coding for LC/C as prepared in pENTRY2 for use in the ~~Gateway~~ GATEWAY[®] (cloning system) site specific recombination cloning system. LC/C has no STOP codon in order to facilitate creation of LC-H_N fusions through recombination.

135 DNA coding for LH_N/C as prepared in pENTRY2 for use in the ~~Gateway~~ GATEWAY[®] (cloning system) site specific

recombination cloning system. LH_N/C has a STOP codon and is thus in the correct format for recombination into an appropriate ~~destination-vector~~ DESTINATION VECTOR (cloning vector).

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DNA coding for LH_N/C as prepared in pENTRY2 for use in the Gateway GATEWAY[®] (cloning system) site specific recombination cloning system. LH_N/C has no STOP codon in order to facilitate creation of LH_N/C-ligand fusions through recombination.

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DNA sequence of a pMTL vector modified to be a suitable ~~destination-vector~~ DESTINATION VECTOR (cloning vector) in which to insert endopeptidase fragments from ~~entry-vectors~~ ENTRY VECTORS (cloning vectors). Vector constructed by insertion of Gateway GATEWAY[®] (cloning system) vector conversion cassette reading frame A into pMAL-c2X. Expression cassette (ptac promoter, male gene, Gateway GATEWAY[®] (cloning system) cassette and polylinker) subsequently cloned into pMTL.

Please replace the second subheading on page 75 with the following:

Example 8 Expression of LH_N/C from a Gateway GATEWAY[®] (cloning system) adapted expression vector.

Please replace the third text paragraph (i.e., the first paragraph following the second subheading), on page 75, with the following:

The LH_NC fragment was cloned into a Gateway GATEWAY[®] (cloning system) ~~entry-vector~~ ENTRY VECTOR (cloning vector) as a *SaII-PstI*. Two version were made with a stop codon within the 3' *PstI* site to terminate the protein at this position (LH_NC STOP; SEQ ID 123/124), or with no stop codon to allow the expression of the fragment with C-terminal fusion partners (LH_NC NS; SEQ ID 131/132). The ~~entry-vector~~ ENTRY VECTOR (cloning vector) was recombined with the ~~destination-vector~~ DESTINATION VECTOR (cloning vector) to allow expression of the fragment with an N-terminal MBP tag. Recombination was according to standard protocols (Invitrogen GATEWAY[®] (cloning system) expression manual).

Please replace the first full paragraph on page 76 with the following:

For expression of the fragment with additional C-terminal domains the LH_NC NS ~~entry-vector~~ ENTRY VECTOR (cloning vector) was recombined with a ~~destination vector~~ DESTINATION VECTOR (cloning vector) carrying additional sequences following the attachment site and in the appropriate frame. The sequence of the DNA encoding the LH_N/C fragment flanked by *att* sites that has the properties necessary to facilitate recombination to create a full fusion is described in SEQ ID 133. For example, the ~~destination-vector~~ DESTINATION VECTOR (cloning vector) pMTL-malE-GW-att-IGF was produced by subcloning the coding sequence for human IGF as an *XbaI-HindIII* fragment into the appropriate sites. Recombination of the LH_N/C NS fragment into this vector yielded pMTL-malE-GW- LH_NC-*att*-IGF.

Please replace the third full paragraph on page 76 with the following:

Those familiar with the art will recognise that a similar approach could be used for other LH_N fragments from either BoNT/C or other serotypes. Similarly other C-terminal purification tags or ligands could be incorporated into ~~destination vectors~~ DESTINATION VECTORS (cloning vectors) in the same way as for IGF above.

Please replace the first subheading on page 76 with the following:

Example 9 Expression of LH_NTeNT from a Gateway GATEWAY[®] (cloning system) adapted expression vector.

Please replace the fourth text paragraph (i.e., the first paragraph following the first subheading), on page 76, with the following:

The LH_NTeNT *Bam*HI-*Hind*III fragment described in Example 7 was subcloned into an ~~entry vector~~ ENTRY VECTOR (cloning vector) to maintain the appropriate reading frames. The ~~entry vector~~ ENTRY VECTOR (cloning vector) was designed to incorporate a factor Xa site immediately adjacent to the *Bam*HI site such that cleavage resulted in a protein starting with the GlySer residues encoded by the *Bam*HI site. The ~~entry vector~~ ENTRY VECTOR (cloning vector) was recombined with a commercially available ~~destination vector~~ DESTINATION VECTOR (cloning vector) carrying an N-terminal 6-His tag (e.g. pDEST17; Invitrogen). The resulting clone pDEST17 LH_NTeNT was expressed in the host strain HMS174 (pACYC tRNA). As described in Example 6. Purification of the fusion protein is also as described in Example 5 with the N-terminal

His tag removed by factor Xa treatment, followed by subsequent removal of factor Xa on a Q-sepharose column.